

UNCLASSIFIED

AD NUMBER

ADB140063

NEW LIMITATION CHANGE

TO

Approved for public release, distribution
unlimited

FROM

Distribution authorized to U.S. Gov't.
agencies only; Proprietary Information;
Dec 1989. Other requests shall be referred
to Commander, US Army Medical Research and
Development Command [Attn: SGRD-RMI-S]
Fort Detrick, Frederick, Maryland
21701-5012.

AUTHORITY

USAMRDC, 28 APR 1993

THIS PAGE IS UNCLASSIFIED

DTIC FILE COPY

L (2)

AD _____

CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND

CLONING OF ITS GENE



ANNUAL REPORT

IVAN I. KAISER

DECEMBER 1989

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6061

University of Wyoming
Department of Molecular Biology
Laramie, Wyoming 82071

Distribution ~~limited~~ to U. S. Gov't agencies only; Proprietary
Information; December, 1989. Other requests for this
document must be referred to the Commander, US Army Medical
Research and Development Command (ATTN: SGRD-RMI-S) Fort
Detrick, Frederick, Maryland 21701-5012.

The findings in this report are not to be construed as an
official Department of the Army position unless so designated
by other authorized documents.

AD-B140 063

90 01 29 059

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

18. REPORT SECURITY CLASSIFICATION Unclassified		19. RESTRICTIVE MARKINGS										
20. SECURITY CLASSIFICATION AUTHORITY		J. DISTRIBUTION/AVAILABILITY OF REPORT Distribution UNLIMITED to U.S. Gov't agencies only; Proprietary Information; Dec 1989										
26. DECLASSIFICATION/DOWNGRADING SCHEDULE												
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)										
6a. NAME OF PERFORMING ORGANIZATION University of Wyoming	6b. OFFICE SYMBOL (if applicable)	7a. NAME OF MONITORING ORGANIZATION										
6c. ADDRESS (City, State, and ZIP Code) Dept. of Molecular Biology Laramie, WY 82071		7b. ADDRESS (City, State, and ZIP Code)										
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development	8b. OFFICE SYMBOL (if applicable) SGRD-RMI-S	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-86-C-6061										
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012		10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A PROJECT NO. 3 M1-61102BS12 TASK NO. AA WORK UNIT ACCESSION NO. 133										
11. TITLE (Include Security Classification) CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND CLONING OF ITS GENE												
12. PERSONAL AUTHOR(S) KAISER, IVAN I.												
13a. TYPE OF REPORT Annual	13b. TIME COVERED FROM 1 Mar 88 TO 30 Nov 89	14. DATE OF REPORT (Year, Month, Day) 1989 December	15. PAGE COUNT									
16. SUPPLEMENTARY NOTATION												
17. COSATI CODES <table border="1"><tr><th>FIELD</th><th>GROUP</th><th>SUB-GROUP</th></tr><tr><td>06</td><td>01</td><td></td></tr><tr><td>06</td><td>03</td><td></td></tr></table>		FIELD	GROUP	SUB-GROUP	06	01		06	03		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) Crotoxin; Presynaptic neurotoxins; rattlesnake toxins; natural toxins; RAI; antitoxins; (←)	
FIELD	GROUP	SUB-GROUP										
06	01											
06	03											
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an <u>in vitro</u> system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs.												
The four monoclonal antibodies and rabbit polyclonals raised against the basic subunit of crotoxin have been further characterized for their cross-reactivity against each other, in the first step of attempting to determine their binding (See Con't)												
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTC USERS		21. ABSTRACT SECURITY CLASSIFICATION Unclassified										
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian		22b. TELEPHONE (Include Area Code) 301-663-7325	22c. OFFICE SYMBOL SGRD-RMI-S									

Epitopes, presynaptic

(19 Con't.)

epitopes. One of the monoclonal antibodies is a potent neutralizer of crot toxin's lethality and phospholipase activity. Another, is a non-neutralizer, but is able to inhibit the phospholipase activity by up to 90%. Work is continuing in an attempt to identify the neutralizing MAb epitope and other antigenic regions in the basic subunit of crot toxin recognized by rabbit polyclonal antibodies. A crot toxin homolog is present in the venom of C. v. concolor, C. s. scutulatus (Type A venom), C. v. vegrandis, and C. d. collilineatus, but none was found in venom from either C. v. lutosus, Lachesis muta muta, or C. atrox/C. atrox-C. s. scutulatus (Type B venom) hybrids. With Lachesis we did demonstrate the presence of a toxic, 60 kd gyroxin-analog, which produced barrel-rolling motions in mice. We have completed the sequence determination of both the basic and acidic subunits of crot toxin. The acidic subunit peptides were difficult, since two of the three peptides were blocked at the amino-terminus by pyroglutamate. Earlier structural studies on crot toxin and related crotalid dimeric, presynaptic neurotoxins indicated that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Additional studies showed however, that when intact crot toxin was exposed to urea and subsequently re-isolated, their spectra were indistinguishable from unexposed, intact crot toxin. Findings which suggest that the spectral changes observed in isolated subunits were artifacts, resulting from structural changes that occurred during subunit isolation by urea ion-exchange that were not easily reversed under our in vitro conditions. Crot toxin cross-linked by a different cross-linker than used by Hendon and Tu, also proved to be non-toxic. These results do not rule out however, the possibility that modification of essential residues resulted in the loss of the observed toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crot toxin. X-ray crystallography studies with purified Mojave toxin from C. s. scutulatus in collaboration with Dr. Keith Ward of the Naval Research Laboratories are progressing. A manuscript describing the initial structural findings is in preparation. Experiments on a synaptosome system for examining choline and acetylcholine using luminometry are continuing. We have encountered numerous technical problems. The first phase on in vitro studies with Dr. Lance Simpson (Jefferson Medical College), on the effects of crot toxin on the isolated phrenic nerve-hemidiaphragm have been completed and are in press. Our cDNA library from C. d. terrificus was screened for clones of both acidic and basic subunit. We were unable to identify any basic subunit clones and the acidic subunit clones that were partially sequenced, had multiple sequence differences. New cDNA and genomic libraries from C. s. scutulatus are in being prepared and screened in collaboration with Dr. Leonard Smith (USAMRIID). Our preparations of ten purified presynaptic neurotoxins have been completed. We have updated the purification procedure for notexin and notechis II-5 from N. s. scutatus and appear to have found and isolated a new, toxic phospholipase from this venom.

SUMMARY

The purpose of this project is to (1) gain greater insight into crototoxin and crototoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an *in vitro* system for examining presynaptic neurotoxin mechanism of action; (3) clone the crototoxin gene as a first step in creating a non-toxic, but immunoreactive crototoxin analog; and (4) explore other possible non-toxic, crototoxin immunogens as potential vaccines against crototoxin and its homologs.

The four monoclonal antibodies and rabbit polyclonals raised against the basic subunit of crototoxin have been further characterized for their cross-reactivity against each other, in the first step of attempting to determine their binding epitopes. One of the monoclonal antibodies is a potent neutralizer of crototoxin's lethality and phospholipase activity. Another, is a non-neutralizer, but is able to inhibit the phospholipase activity by up to 90%. Work is continuing in an attempt to identify the neutralizing MAb epitope and other antigenic regions in the basic subunit of crototoxin recognized by rabbit polyclonal antibodies. A crototoxin homolog is present in the venom of C. v. concolor, C. s. scutulatus (Type A venom), C. vegrandis, and C. d. collilineatus, but none was found in venom from either C. v. lutosus, Lachesis muta muta, or C. atrox/C. atrox-C. s. scutulatus (Type B venom) hybrids. With Lachesis we did demonstrate the presence of a toxic, 60 kd gyroxin-analog, which produced barrel-rolling motions in mice. We have completed the sequence determination of both the basic and acidic subunits of crototoxin. The acidic subunit peptides were difficult, since two of the three peptides were blocked at the amino-terminus by pyroglutamate. Earlier structural studies on crototoxin and related crotalid dimeric, presynaptic neurotoxins indicated that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Additional studies showed however, that when intact crototoxin was exposed to urea and subsequently re-isolated, their spectra were indistinguishable from unexposed, intact crototoxin. Findings which suggest that the spectral changes observed in isolated subunits were artifacts, resulting from structural changes that occurred during subunit isolation by urea ion-exchange that were not easily reversed under our *in vitro* conditions. Crototoxin cross-linked by a different cross-linker than used by Hendon and Tu, also proved to be non-toxic. These results do not rule out however, the possibility that modification of essential residues resulted in the loss of the observed toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crototoxin. X-ray crystallography studies with purified Mojave toxin from C. s. scutulatus in collaboration with Dr. Keith Ward of the Naval Research Laboratories are progressing. A manuscript describing the initial structural findings is in preparation. Experiments on a synaptosome system for examining choline and acetylcholine using luminometry are continuing. We have encountered numerous technical problems. The first phase of *in vitro* studies with Dr. Lance Simpson (Jefferson Medical College), on the effects of crototoxin on the isolated phrenic nerve-

hemidiaphragm have been completed and are in press. Our cDNA library from C. d. terrificus was screened for clones of both acidic and basic subunit. We were unable to identify any basic subunit clones and the acidic subunit clones that were partially sequenced, had multiple sequence differences. New cDNA and genomic libraries from C. s. scutulatus are in being prepared and screened in collaboration with Dr. Leonard Smith (USAMRIID). Our preparations of ten purified presynaptic neurotoxins have been completed. We have updated the purification procedure for notexin and notechis II-5 from N. s. scutatus and appear to have found and isolated a new, toxic phospholipase from this venom.



Accession For	
NTIS	CRAN
DTIC	TAB
Unpublished	
Justification	
By _____	
Distribution /	
Availability Dates	
Dist	Avail. and/or Special
B-3	

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

TABLE OF CONTENTS

Summary.....	1
Foreword.....	3
List of Figures and Tables.....	5
Body of Report	
Statement of the Problem.....	6
Background and Approach to the Problem.....	6
Results	
I. Monoclonal Antibodies.....	10
II. Effect of monoclonal antibodies on phospholipase activity.....	12
III. Polyclonal antibodies.....	13
IV. Crototoxin Homologs	
(A) <u>Crotalus durissus collilineatus</u>	13
(B) <u>Crotalus atrox</u> and <u>Crotalus atrox-C. s. scutulatus</u> hybrids.....	13
V. Sequencing of crototoxin	
(A) Basic subunit.....	14
(B) Acidic subunit.....	14
VI. Spectral Measurements.....	14
VII. Myotoxins.....	15
VIII. Gyroxin Analog.....	15
IX. Cross-linking of crototoxin subunits.....	15
X. Cloning.....	16
XI. X-Ray Crystallography of Mojave toxin.....	16
XII. Neurotoxicity mechanism of action.....	16
XIII. Purified toxins.....	18
Discussion.....	18
Conclusions.....	21
Recommendations.....	22
Literature Cited.....	23
Figures.....	26
Tables.....	29
Distribution List.....	30

LIST OF FIGURES

- Figure 1. Competition of antibody binding to the basic subunit of crot toxin.....26
Figure 2. Competition of MAb binding with polyclonal antibodies.....26
Figure 3. Competition of MAbs and polyclonal Ab for biotinylated-crot toxin.....26
Figure 4. Phospholipase activity of crot toxin on phosphatidylcholine.....26
Figure 5. Phospholipase activity of crot toxin in the absence and presence of MAb 1.....27
Figure 6. Phospholipase activity of phospholipase A₂ from *C. atrox*.....27
Figure 7. Phospholipase activity of crot toxin in the presence of MAb 2 and 5.....27
Figure 8. Phospholipase activity of the basic subunit of crot toxin.....27
Figure 9. Phospholipase activity of the basic subunit of crot toxin.....28
Figure 10. Phospholipase activity of the basic subunit of crot toxin

LIST OF TABLES

- Table 1. Summary and semiquantitation of competition binding data.....29

BODY OF REPORT

STATEMENT OF PROBLEM

The purpose of this project is to (1) gain greater insight into crototoxin and crototoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an *in vitro* system for examining presynaptic neurotoxin mechanism of action; (3) clone the crototoxin gene as a first step in creating a non-toxic, but immunoreactive crototoxin analog; and (4) explore other possible non-toxic, crototoxin immunogens as potential vaccines against crototoxin and its homologous crototoxin analogs.

BACKGROUND AND APPROACH TO THE PROBLEM

The discovery of crototoxin, a potent, enzymatic neurotoxin from the venom of the South American rattlesnake (*Crotalus durissus terrificus*) by Slotta and Fraenkel-Conrat (1) marked the beginning of modern-day snake venom research. Crototoxin is a heterodimeric protein, consisting of a moderately toxic basic phospholipase A₂ and a acidic, non-toxic subunit composed of three small proteins (2). The acidic subunit is required for full toxicity, but has no other identified function. Evidence gradually accumulated suggesting similarity between crototoxin, Mojave toxin from *Crotalus s. scutulatus*, concolor toxin from *Crotalus viridis concolor*, and vegrandis toxin from *Crotalus vegrandis*, although disconcerting differences persisted (see ref. 3 and 4 for review). From our early investigations (5), we were reasonably certain that the above four toxins were similar structurally and functionally. We wanted to extend these studies and prepared rabbit antiserum against crototoxin, concolor toxin, and Mojave toxin, as well as against the acidic and basic subunits of crototoxin. These antisera were used to examine the antigenic relatedness of purified crotalid toxins and subunits by both double immunodiffusion and ELISA. We also determined the relative efficacy of antisera raised against the subunits and intact complex of crototoxin in neutralizing the toxicity of crototoxin and related toxins in mice (6). At the same time we prepared monoclonal antibodies to crototoxin. These have provided us with additional tools to probe crototoxin structure and we were fortunate to generate a neutralizing monoclonal antibody, which may provide for either the development of an anti-idiotype vaccine, or the identity of a neutralizing antigenic site on crototoxin (7,8). We have now partially characterized four different MAbs raised to the basic subunit of crototoxin, with respect to their epitope binding sites on crototoxin. Their effects on crototoxin's phospholipase activity and neurotoxicity have also been probed. Studies with the MAbs are continuing.

We have also examined the venom from the Great Basin rattlesnake (*C. v. lutosus*), the Uracan rattlesnake (*C. vegrandis*), the Brazilian *C. d. collilineatus*, and the Western

diamondback rattlesnake (*C. atrox*), as well as Western diamondback-Mojave rattlesnake (*C. s. scutulatus*) hybrids, for crototoxin-like neurotoxins. One publication has resulted on the Uracoan study (Kaiser and Aird, 9); one on the Great Basin rattlesnake (Aird, Seebart, and Kaiser; 10), the Western Diamondback results have just been published (Aird, Thirkhill, Seebart, and Kaiser; 11), and the *C. d. collilineatus* results are in preparation. Only in the two South American rattlesnakes, the Uracoan and *C. d. collilineatus* did we find crototoxin homologs.

Crototoxin has been the most extensively studied and characterized rattlesnake neurotoxins. When this work was started, the amino acid sequence of its basic subunit was largely determined, with the exception of the Asp/Asn and Glu/Gln residues, which could not be differentiated (12). We completed the sequence of the basic subunit, and have published those results (Aird, Kaiser, Lewis, and Krugel; 13 and 14). All three acidic subunit chains have now been sequenced and the results have either been published or submitted for publication (2, 15, 16). Repeated attempts to sequence the blocked, amino-terminal end of the B-chain by conventional methods were unsuccessful. In 1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. He was recently able to provide us with the amino-terminal sequence of the B-chain. Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution, since others unsuccessfully attempted to sequence the peptides for the past fifteen years.

Both subunits of crototoxin have sequence homology with phospholipases A₂ even though the acidic subunit consists of three separate chains linked by disulfide bonds. Only the basic subunit however, which consists of a single polypeptide chain, actually manifests phospholipase activity.

A very striking conformational change reportedly occurs in crototoxin upon complex formation (17). Marked changes in both fluorescence and circular dichroism (CD) spectra were reported by Hanloy (17) to appear when the subunits interact, suggesting a large change in the structure of either one or both subunits. In contrast, when the homologous toxin from *C. s. scutulatus* venom (Mojave toxin) was examined, no evidence for such a conformational change was noted (Tu et al., 18). Estimates of secondary structure for these proteins conflict with x-ray crystallographic data from non-toxic, homodimeric phospholipases. In general, phospholipases A₂ appear to have approximately 50% alpha-helical structure (19), while estimates from CD and Raman studies give values of 12-18% and 70% for the *C. d. terrificus* and *C. s. scutulatus* basic subunits, respectively. Thus there is an apparent conflict in the literature regarding the existence of both conformational changes upon complex formation and the secondary structure of the subunits.

To resolve these uncertainties, we examined the spectral properties of purified neurotoxins and their subunits from four crotalid taxa, using CD, Fourier Transform infrared (FTIR), and fluorescence spectroscopy. We also found evidence that could suggest conformational changes in

the isolated subunits upon dissociation in all cases examined and evidence that the secondary structure of these toxins differed from those predicted for non-toxic venom and pancreatic phospholipases A2. Further studies of crototoxin, however, suggested that the apparent conformation changes arose primarily from the presence of urea during the subunit isolation process (20).

Samples of purified Mojave toxin have been provided to the laboratory of Dr. Keith Ward (Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C.) for crystallization studies. His laboratory has been successful in obtaining crystals suitable for x-ray diffraction studies (Norden *et al.*, 21). Detailed x-ray structural analysis should provide valuable insights into rattlesnake presynaptic neurotoxin structure.

Previous work by Hendon and Tu (22) was designed to examine whether dissociation was essential for neurotoxicity. Dimethyl suberimidate (DMS), was used to covalently bind the two subunits. Their data suggested that they introduced an average of three crosslinks per complex. At least one of these must have been between subunits because recovered complex could not be dissociated in 6M urea. Sequence analyses indicate the presence of 10 lysine residues (the most likely reactant) in the basic subunit and one each in the A-chain and B-chain of the acidic subunit (2). The DMS-crototoxin had comparable levels of phospholipase A₂ activity to that of unmodified crototoxin (21 $\mu\text{mol}/\text{min}\cdot\text{mg}$). LD₅₀ values in mice increased from 0.06 $\mu\text{g}/\text{g}$ to >1.5 $\mu\text{g}/\text{g}$. Retention of phospholipase activity and loss of neurotoxicity in the cross-linked complex was interpreted to reflect "interference between the cross-linked complex and the target site." Recent results from three different groups indicate that loss of neurotoxicity may have resulted from modification of the ϵ -amino group of lysine and not necessarily cross-linking. Using chemical derivatization techniques, Rosenberg's group (23) observed that lysine or arginine group modification in basic phospholipase A₂ enzymes, frequently results in greater loss of pharmacological than of enzymatic activity. They note that modification of these basic amino acids may alter the protein's stability, distribution, or tissue binding ability. Jeng and Fraenkel-Conrat (24) also observed inactivation of neurotoxicity in crototoxin upon acetylation of amino groups and suggested that the inactivation was due to a "discrete change in the conformation of the molecule induced by the loss of positive charges." In work with biotinylated-crototoxin, we observed that biotinylation using N-hydroxysuccinimido-biotin resulted in a greater than 15-fold decrease in the LD₅₀. Since both the activated biotin and DMS react primarily with ϵ -amino groups of lysine, the results suggested that loss of toxicity observed by Hendon and Tu (22), might have resulted from loss of essential amino side chains and be unrelated to cross-linking per se.

Hendon and Tu also used DEAE-cellulose column chromatography to separate cross-linked and unreacted toxin components in phosphate-buffered urea. Their neurotoxicity loss may have resulted, in part, from carbamylations during chromatography resulting from spontaneous breakdown of urea generating cyanate which reacts with amino groups. Their unreacted

controls were not cycled over urea columns. We repeated Hendon and Tu's cross-linking experiments with the appropriate controls, and screened a number of different cross-linkers. The most effective was found to be a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which activates carboxyl chains, thereby enabling strategically located ϵ -amino groups of lysine to form internal covalent links in the absence of any "linker" (25). Water soluble carbodiimides can also react with hydroxy groups on tyrosines, but these are presumably removed by hydroxylamine treatment (26). Cross-linked crot toxin was isolated by gel filtration in the presence of urea and a number of its properties examined.

We have also examined the myotoxin fraction from *C. v. concolor*. Goncalves (27) reported the presence of a highly basic polypeptide from the venom of *C. d. terrificus*, which he named crotamine. Since that time, crotamine-like proteins have been reported in the venoms of a number of different crotalids. Although the exact biological mode of action of these myotoxins is not known, it is clear that their first microscopically observable effect is on muscle cells, causing vacuolation (Ownby *et al.* 28). The purpose of the work by us was to determine whether the minor structural differences between isotoxins isolated from one batch of venom resulted in any biological and immunological differences. This has implications to our work on crot toxin, because of the crot toxin and crot toxin-homolog isoforms recently identified by us and others (9,29).

We thought that at this stage of our research, that we would have available an *in vitro* system of synaptosomes and/or tissue culture cells with well defined responses to crotalid neurotoxins. We have conducted experiments using guinea pig brain synaptosomes and ^3H -choline uptake and release measurements to monitor responses to crotalid neurotoxins. Existing assays and procedures have proven to be more extensive and involved than originally anticipated. New methodology, involving luminometry is being examined and attempts are being made to use this method in conjunction with synaptosomes as a system for studying presynaptic neurotoxin action *in vitro*.

We have also initiated a collaboration with Dr. Lance Simpson (Jefferson Medical College), who has examined crot toxin for its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. We have probed the action of crot toxin, using procedures that Simpson has employed successfully with clostridial neurotoxins. In work to date, we have added the intact toxin or the individual subunits to the isolated phrenic nerve-hemidiaphragm preparation. This was done in the presence or absence of drugs or procedures known to alter the progression of clostridial neurotoxin-induced paralysis. Results have provided additional insights into the mechanism of action of phospholipase A₂ neurotoxins (see ref. 30).

Our progress involving the cloning of the crot toxin gene has been fraught with difficulties.

We have had several false starts with cDNA clones from C. d. terrificus. We amplified cDNA using the polymerase chain reaction, inserted the product into Bluescript II KS plasmids and transformed E. coli. Partial sequencing of several clones indicated that they contained acidic subunit cDNA. In addition, all sequences had base changes that may have been introduced during the polymerase chain reaction amplification. We were also unsuccessful in identifying any clones that contained basic subunit cDNA. Work is continuing with new cDNA and genomic libraries from C. s. scutulatus, in collaboration with Dr. Leonard Smith at USAMRIID.

We have completed our isolation and purification of 100 mg of each of ten different presynaptic neurotoxins. These have been provided to Dr. John Middlebrook at USAMRIID. Toxins isolated included pseudexin, notexin, taipoxin, textilotoxin, β -bungarotoxin, concolor toxin, ammodytoxin, caudoxin, Mojave toxin and vegrandis toxin.

R E S U L T S

I. MONOCLONAL ANTIBODIES. A manuscript describing the preparation of a crotoxin neutralizing monoclonal antibody and three non-neutralizing monoclonal antibodies has been published (Kaiser and Middlebrook, 7). These four different monoclonal antibodies (MAbs) all typed as IgG₁ subclass and were raised against the basic subunit of crotoxin. The neutralizing antibody could neutralize \approx 1.6 moles of purified intact, crotoxin per mole of antibody and enhanced the neutralizing ability of commercial polyvalent crotalid antivenom against the lethality of crude C. d. terrificus venom four-fold. Paradoxically, this monoclonal antibody by itself was ineffective against the lethality of crude C. d. terrificus venom. Using an enzyme-linked immunosorbent assay (ELISA), we tested various proteins for competitive inhibition of binding of biotinylated-crotoxin to plates coated with the four individual MAbs. Concolor toxin, vegrandis toxin, intact crotoxin, Mojave toxin, and the basic subunit of crotoxin showed increasing effectiveness as displacers of crotoxin from the neutralizing MAb. None of the MAbs reacted with purified phospholipase A₂ enzymes from Crotalus atrox or Crotalus adamanteus, nor any of the components present in the crude venoms from four different elapids known to contain presynaptically acting neurotoxins, which show some sequence identity to crotoxin.

As a first step in our epitope analysis for the four MAbs, we examined each in two different types of ELISAs. One involved labeling each MAb with biotin and determining how each individual biotin-labeled MAb competed for the same binding site on its antigen with one of the other MAbs. Alternatively, one of the MAb can be immobilized and a second MAb allowed to first incubate with biotinylated-antigen which was then reacted with immobilized MAb.

Results for the epitope analysis of biotinylated MAb 1, is shown in Fig. 1. In regard to only MAbs, the homologous, non-biotinylated MAb is the most effective competitor in each assay, lending credibility to the assay. Each MAb also appears to have a unique specificity. A summary and semiquantitation of the data are presented in Table 1, where the interaction between the

four MAbs are denoted by + signs. The greater the number of +, the greater the interaction observed. Included in Table 1 are rabbit polyclonal antibodies (P) raised against the basic subunit of crot toxin. In all cases the polyclonal antibodies were a more potent inhibitor of biotinylated-MAb than the unmodified, homologous MAb. Polyclonal antibodies corresponding to the epitope recognized by the MAb are probably present in the Ab mixture, plus additional Ab that recognize adjacent and/or overlapping epitopes. Some MAb could therefore be inhibited by steric inhibition of bound polyclonal antibodies--in certain instances at great distance from the antigenic site. Rabbit polyclonal Abs also appear to recognize additional epitopes. In one experiment rabbit polyclonal antibodies raised to the basic subunit of crot toxin was biotinylated and an inhibition experiment performed with a mixture of all four MAbs. Figure 2 shows that the inhibition was limited to about 60%, indicating that rabbit polyclonal antibody recognized additional epitopes on the basic subunit antigen. Presumably then, only a fraction of all possible epitopes are represented by our four MAbs, assuming the original mouse antisera was equivalent to the rabbit antisera.

In a complementary assay, wells are coated with a MAb and biotinylated-crot toxin is mixed in a separate tube containing different dilutions of the test MAb. After overnight incubation, the biotinylated-crot toxin-MAb mixture is added to the wells. If the antigenic site is still available, then significant binding of the biotinylated-crot toxin should occur and subsequently be detected by the avidin-peroxidase conjugate. Epitope analysis using biotinylated-crot toxin and MAb 1 coated plates is illustrated in Fig. 3 and summarized in a semiquantitative manner in Table 2. There is excellent correlation between these results and those of the earlier described complementary experiment. This suggests that the biotinylation of either crot toxin or antibody did not significantly affect its binding. Nor in the solid-phase assays does there appear to be conformational changes in either the crot toxin or antibody that affects binding. In these assays, the role of crot toxin and antibody have been reversed which permits the affects of derivatization and binding to be examined. Polyclonal antibodies were again potent competitors for each of the MAbs.

We have also now completed western blots of crot toxin and related proteins from reducing and non-reducing SDS-PAGE gels, using rabbit polyclonal antibodies raised to intact crot toxin and the four MAbs as primary antibodies. Polyclonal antibodies were purified on a crot toxin-Sepharose affinity column and the four MAbs on protein-A columns. Blotting methods were essentially as described (31) with either goat-antirabbit or goat-antimouse conjugated with peroxidase serving as secondary antibodies.

We observed strong signals from the basic subunit of crot toxin with all Abs, whether applied as crude venom, intact crot toxin, or purified subunit--in the absence of reducing agent. When DTT was included in the protein solubilizing solution, strong basic subunit bands were observed with the polyclonal Ab and MAb 11. Progressively weaker signals of the basic subunit were observed with reduced samples with MAbs 2, 1, and 5 respectively.

II. EFFECT OF MONOCLONAL ANTIBODIES ON PHOSPHOLIPASE ACTIVITY. Phospholipase activity is associated with all presynaptic neurotoxins. It was therefore of interest to examine the effects that the neutralizing and non-neutralizing MAbs would have on the phospholipase activity of intact crot toxin, its basic subunit, and a related, non-toxic phospholipase from C. atrox.

Preincubation of monoclonal antibody 1 (MAb 1) with crot toxin at a molar ratio of 1:1, resulted in a potent inhibition of crot toxin's phospholipase activity as shown in Fig. 4. At lower antibody to crot toxin ratios, the inhibition was relaxed somewhat, although even at a 0.125:1 ratio the rate of phospholipid hydrolysis was inhibited by about 25%. By increasing antibody to crot toxin ratios from 1:1 to 4:1 the inhibition was slightly enhanced, although in all cases it was generally greater than 90%. Even in experiments where MAb 1 was added to an ongoing reaction containing crot toxin, phospholipid, and 1% Triton-X100, there was an immediate inhibition of phospholipase activity (Fig. 5). This indicates that the enzyme is available or becomes available for reaction with the antibody in the presence of substrate.

Crotalus atrox phospholipase A₂ at an antibody to phospholipase molar ratio of 4:1 showed no evidence of inhibition, as may be seen in Fig. 6. This is consistent with the lack of competition shown by this protein for the binding of biotinylated intact crot toxin to MAb 1 and also in ELISAs using immobilized antigens.

Monoclonal antibody 2, while not a neutralizing antibody, showed inhibition of crot toxin's lipase activity similar to MAb 1 (Fig. 7A). Monoclonal antibodies 5 (Fig. 7B) and 11 (not shown), both displayed partial inhibition of crot toxin's lipase activity but substantially less than lines 1 and 2.

We were surprised to find that unlike intact crot toxin, phospholipase activity associated with the isolated basic subunit was not inhibited well under the same conditions that led to potent inhibition of intact crot toxin phospholipase activity (preincubation at a 1:1 molar ratio) with MAb 1. Fig. 8 illustrates this difference in inhibitory susceptibility, and should be compared with Fig. 4. In different experiments, we observed from 30-50% inhibition of basic subunit phospholipase activity, when preincubated with MAb 1 at a 1:1 molar ratios. This suggested that the presence of the acidic subunit was necessary for potent inhibition by MAb 1. To examine the role of the acidic subunit further, basic subunit was mixed with MAb 1 and preincubated 15' in the absence and presence of a four-fold excess of acidic subunit relative to the basic subunit. The increased phospholipase inhibition in the presence of the acidic subunit is dramatic (Fig. 9). In Fig. 5 we observed that addition of MAb 1 to an ongoing reaction of intact crot toxin hydrolyzing phospholipid substrate led to an immediate decrease in its hydrolytic activity. We observed a similar response when a four-fold excess of acidic subunit over basic subunit was added to an ongoing phospholipase assay (Fig. 10). There was an abrupt inhibition of phospholipase activity upon acidic subunit addition.

III. POLYCLONAL ANTIBODIES. Polyclonal rabbit antisera were raised against ten snake phospholipase A₂ neurotoxins and one snake phospholipase A₂ cytotoxin. Immunological cross-reactivities between these toxins, two other snake phospholipase A₂ enzymes and pancreatic phospholipase A₂ were studied using ELISA technology. All snake phospholipase A₂ neurotoxins fell into two main antigenic classes. One antigenic class was composed of all the elapid toxins tested (textilotoxin, taipoxin, notexin, pseudexin and β -bungarotoxin), the cytotoxic phospholipase A₂ from Naja naja atra and pancreatic phospholipase A₂. Beta-bungarotoxin seemed to be in an immunological subclass of its own compared to the rest of the elapid toxins. The second antigenic class was comprised of crotalid and viperid phospholipase A₂ neurotoxins (crototoxin, concolor toxin, Mojave toxin, vegrardis toxin, ammodytoxin and caudoxin). Our data indicated that the viperid toxins, caudoxin and ammodytoxin, were immunological subclass apart from the crotalid toxins. In all cases, the homologous antisera were effective in protecting mice from the lethal effects of the toxins. In certain instances heterologous antisera could also protect. These data suggest that the snake phospholipase A₂ neurotoxins have common epitopes and, in some instances, common neutralizing epitopes. Additional details of this work may be found in reference 32 by Middlebrook and Kaiser.

IV. CROTOXIN HOMOLOGS. (A) CROTALUS DURISSUS COLLILINEATUS. In their paper on isoforms of crototoxin, Faure and Bon (29) reported that crototoxin-like material "was a minor component or even absent in venoms" from C. d. collilineatus. Contrary to this report, we found significant amounts of crototoxin-like material in three different lots of C. d. collilineatus. In fact, all samples showed that the crototoxin-like material represented more than 40 % of the total venom protein. In one lot it approached 80 %. The crototoxin-like material was isolated and compared with crototoxin using a variety of techniques, including gel filtration on Sephadryl S-200SF, SDS-PAGE, isoelectric focusing, phospholipase activity, lethality, immunological reactivity and anion-exchange chromatography. All results indicated that the principal toxic component in C. d. collilineatus venom was a homolog of crototoxin. A manuscript describing this work is in preparation.

(B) CROTALUS ATROX AND CROTALUS ATROX-CROTALUS SCUTULATUS SCUTULATUS HYBRIDS. This work was outlined in last year's report and was recently published in the Journal Of Herpetology (11). Venom composition and morphology of a male C. atrox, a female C. s. scutulatus, their F1 hybrid offspring, and a half sibling C. s. scutulatus were examined. F1 hybrids showed morphological and venom characteristics of both parental species. F2 hybrids showed morphological characteristics of both C. atrox and C. s. scutulatus. C. s. scutulatus venom (Type B) was shown to be qualitatively similar to that of C. atrox and qualitatively very different from C. s. scutulatus (Type A) based on gel filtration and ion-exchange chromatography, ELISA and PAGE. Guidelines were provided regarding the detection of

presynaptic neurotoxins in crotalid venoms to prevent their being confused with non-toxic phospholipases A₂. Several hypotheses were offered to explain the origin of the Type B venom population within C. s. scutulatus. This work has practical importance from the standpoint of venom neutralization using crotalid antisera.

V. PROTEIN SEQUENCING. CROTOXIN. (A) BASIC SUBUNIT. Determination of the primary sequence of the basic subunit has been completed. See year 1 report and references (13, 14) in Literature Cited.

(B) ACIDIC SUBUNIT. A partial sequence was reported in year 1 annual report and in reference 2. A preliminary sequence for the amino-end of the B-chain was reported in an abstract (15) and a manuscript describing the details of the sequence determination has been submitted for publication (16). The N-terminus was blocked by pyroglutamate, although the residue was refractory to the enzyme pyroglutamate aminopeptidase. The sequence was determined by a combination of Edman degradation and tandem mass spectrometry, in collaboration with Dr. Don Hunt at the University of Virginia. The B-chain contained 35 amino acids and showed 91 % amino acid identity with the corresponding segment from Mojave toxin, a homologous neurotoxin from C. s. scutulatus, and is shown below.

pEEDGEIVCGEDDPCTQICG/ECDKAAAICFRNSMDT

The sequence of the last 24 residues of the B-chain was consistent with that previously published (2), except at position 20, where Edman degradation gave glycine and mass spectrometry gave glutamic acid.

VI. SPECTRAL MEASUREMENTS. A preliminary report on the spectroscopic studies of crotoxin and its homologs has been made (33). Over the past year additional studies have been carried out, which have led to substantially different conclusions than were reached earlier. Briefly, structures of four related neurotoxins and their purified subunits from the venoms of C. d. terrificus, C. vegrandidis, C. s. scutulatus, and C. viridis concolor were examined by circular dichroism (CD), deconvolution Fourier-transform infrared (FTIR), and fluorescence spectroscopy. CD spectra suggest that in general, the isolated subunits were decreased slightly in α -helix, while increased in β -sheet structure, relative to intact toxins. These results were consistent with FTIR results. Fluorescence quenching (50-80%) was also observed in three of the four intact toxins as compared to spectra predicted by summation of free acidic and basic subunit spectra. It was tempting to conclude from these results that major conformational changes occur in individual subunits upon formation of the dimeric toxins. Intact crotoxin, however, when exposed to urea and then reisolated, yields spectra (CD, FTIR, and fluorescence) that are virtually identical to control intact crotoxin. These findings suggest that the enhanced fluorescence exhibited by the isolated subunits, as well as the secondary structural changes in

α -helix and β -sheet, are artifacts resulting from structural changes that occur during subunit isolation by urea ion-exchange chromatography, which are not reversible under our *in vitro* conditions of subunit assembly. In spite of these structural changes, LD₅₀-values of intact crot toxin reassembled from isolated subunits are unaltered from those of native crot toxin. This work has just been published (20).

VII. MYOTOXINS. The physiological and immunological properties of small myotoxins from the venom of the midget faded rattlesnake (*C. v. concolor*) has now been published. All four myotoxin subfractions showed immunological identity with myotoxin a from *C. v. viridis* and myotoxin a antiserum. The more basic concolor myotoxin was about twice as lethal to mice as the less basic myotoxin. Please see reference 34 for details.

VIII. GYROXIN ANALOG. Clinical observations of possible neurotoxic activity in bushmaster (*Lachesis muta muta*) envenomations, coupled with the accepted ancestral relationship of *Lachesis* to other crotalids, suggested that *Lachesis* venom might contain a crot toxin-like molecule. Crude venom and gel-filtration fractions showed modest reactivity in ELISAs using rabbit polyclonal antibodies raised against the basic subunit of crot toxin, but no reaction was detected with a murine monoclonal antibody raised against the same antigen. Phospholipase assays, LD₅₀ determinations, and SDS-polyacrylamide gel electrophoresis indicated the presence of non-toxic phospholipases, but no crot toxin homologs. A higher molecular weight, toxic protein (60,000) with an LD₅₀ of 0.07 μ g/g in mice was isolated and purified, which induced gyroxin-like, rapid rolling motions in mice. Its amino-terminal sequence shows considerable amino acid sequence identity with gyroxin from the venom of *C. d. terrificus* and other serine proteases. A manuscript detailing this work has been published in *Toxicon* (35).

IX. CROSS-LINKING OF CROTOXIN SUBUNITS. A manuscript entitled "Effects of subunit cross-linking on the properties of crot toxin" has been accepted for publication (36). In this paper crot toxin was covalently cross-linked using the water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Gel filtration of the reaction mixture in 8M urea gave rise to several peaks not observed with native crot toxin and permitted separation of cross-linked crot toxin. This material had the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and an 80% reduction of reactable lysine residues. It was also non-toxic, had reduced immunological cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crot toxin and had lost >95% of its phospholipase activity. This work is in keeping with the results of Hendon and Tu (22), which suggested that subunit dissociation was required for expression of neurotoxicity. It does not, however, rule out the possibility that the loss of toxicity is due to the modification of essential residues rather than subunit cross-linking.

X. CLONING. Messenger RNA isolated from the venom gland of C. d. terrificus, was reverse transcribed. This product was amplified using the polymerase chain reaction and cloned into Bluescript II KS plasmids. Clones with inserts were partially sequenced, using the double-stranded method. Initially we had technical problems with the sequencing as a result of extensive higher ordered structure that was giving us hesitation points in the sequencing ladder. Reaction conditions were adjusted to compensate for these difficulties. We found however, that all clones were acidic subunit clones. About six of these were partially sequenced and they were clearly acidic subunit clones, except that they all showed numerous sequence differences. It was unclear whether these represent different gene products or whether we inadvertently introduced these differences during the amplification reaction. In view of sequence differences and the absence of any apparent basic subunit clones, we decided to shelve our work with C. d. terrificus and focus on cDNA and genomic libraries from the tissues of the Mojave rattlesnake (C. s. scutulatus). A cDNA library is currently being prepared by Dr. Leonard Smith, USAMRIID, and we will prepare a genomic library from liver tissue in our laboratory. Screening of the cDNA library for acidic and basic subunit sequences is in progress. Subcloning, sequencing and expression of clones will be coordinated between Smith's laboratory and ours. We are hopeful that these studies will help resolve some of the questions that have been generated during our work with crotalid neurotoxins.

XI. X-RAY CRYSTALLOGRAPHY OF MOJAVE TOXIN IN COLLABORATION WITH KEITH WARD. Mojave toxin from the Mojave rattlesnake (C. s. scutulatus) has been isolated, purified, and crystallized in a form suitable for X-ray diffraction analysis. The crystals are orthorhombic, exhibit the symmetry of space group $P2_12_12_1$, and have lattice constraints: $a = 38.6$, $b = 69.9$, and $c = 77.6 \text{ \AA}$. Over 11,000 items of reflection data have been collected to a resolution of 2.1 \AA . The data have been phased by the molecular replacement method using as a model the structure of dimeric phospholipase A₂ from C. atrox. An atomic model has been refined using Konnert-Hendrickson restrained refinement. The current $R = 0.32$. The basic phospholipase subunit is related to the nontoxic 'chaperon' subunit by an approximate dyad axis, and is very similar in structure to the C. atrox phospholipase subunit. The relative structural disposition of the three chains of this subunit to that of the phospholipase subunit can now be approximated. This structural information should provide important clues to the mechanism of action of this neurotoxin. A manuscript describing this phase of the work is in preparation.

XII. NEUROTOXICITY MECHANISM OF ACTION. (A) Methods for the estimation of acetylcholine (ACh) and choline (Ch) have been evolving since the early 1900's. As they evolved, the sensitivity of these methods have increased from detecting micromolar to picomolar quantities. Methods have ranged from bioassays, to techniques of colorimetry, spectrophotometry, gas chromatography, HPLC, mass spectroscopy, and radioenzymatic assays. Israel and Lesbats (37)

reported a chemiluminescence method for the assay of ACh. This procedure couples ACh to a series of enzymatic reactions with the eventual production of light. A photomultiplier measures the amount of light produced, which is related to the amount of original ACh present. We spent about six months on this methodology, hoping to be able to monitor the continuous release of ACh from guinea pig brain synaptosomes as reported in the literature. To date, the methodology has proved to be irreproducible in our hands.

None of our studies have revealed the source of irreproducibility. Enzyme ratios and pH influence the chemiluminescence reaction, but do not appear to play a major role in variable results. Neither did changing of the intensity of room lighting, source of either buffer or enzymes, injection method, changing of oxidizing agent concentration, and other assay modifications. Comparison of our borrowed Model 20 Turner Designs luminometer with another instrument of the same type shows no great differences. We have also had LKB provide us with one of their "loaner" luminometers, which apparently had traveled to one to many exhibitions and had numerous electronic and mechanical problems. We are currently re-evaluating the luminometry procedure as it applies to the determination of ACh and Ch.

(B) Crototoxin and its two subunits were examined for their neuromuscular blocking activity on the phrenic nerve hemidiaphragm preparation in collaboration with Dr. Lance Simpson. This work has been accepted for publication (30). Briefly, two types of experimental approaches were used, the first of which separated the toxin binding step from subsequent events in paralysis and the second of which did not. In both paradigms, the toxin produced concentration-dependent blockade of transmission. However, the results with low concentrations were variable, and in some cases complete neuromuscular blockade did not develop. The isolated acidic and basic subunits possessed little toxicity. In experiments designed to characterize binding, the intact toxin displayed the following properties: i) the apparent half-time for tissue association was about 22 min, ii) binding was not affected by low temperature, the presence or absence of nerve stimulation, and the substitution of strontium for calcium, and iii) when binding was allowed to go to completion, reversibility was negligible. Pretreatment of tissues with the isolated subunits of crototoxin did not enhance or inhibit the binding of the parent molecule. Modification of one histidine residue in the isolated basic subunit, followed by reconstitution with unmodified acidic subunit, generated a molecule with only about 10% of the activity of the native toxin. The modified toxin could not be used to antagonize binding of the native toxin. Both polyclonal and monoclonal antibodies were generated that neutralized the biologic activity of crototoxin. In experiments that separated the binding step from later events in paralysis, the polyclonal preparation continued to locate and partially neutralize tissue-bound toxin. In experiments that initiated events that follow binding, polyclonal antibodies were progressively less effective with time in neutralizing toxin. The monoclonal preparation did not neutralize toxin after the binding step was complete.

XIII. PURIFIED TOXINS. During the past year or so, we have been involved in the purification of ten different snake venom presynaptic neurotoxins. About 100 mg of each was prepared. These correspond to monomeric (pseudexin, notexin, ammodytoxin, caudoxin), dimeric (β -bungarotoxin, concolor toxin, Mojave toxin, vegrandis toxin), trimeric (taipoxin), and pentameric (textilotoxin) toxins. Availability of these purified samples will enable us to now carry out a number of comparative experiments with crot toxin that were not possible previously, such as those described in reference 32. We expect to use some of this material for studies involving the pharmacology and functionality of this class of snake venom toxins, comparative biochemistry, physical and structural investigations, and immunology.

DISCUSSION

Among the MAbs, the homologous MAb was its own best inhibitor; rabbit polyclonal antibodies were at least as effective. Although not particularly strong, the best competitor for MAb 1 was MAb 5, with 2 and 11 showing little competition. MAbs 2 and 11 also showed little competition for 5. MAb 2 and 11 were strong competitors with each other.

These findings suggest that these three MAbs may recognize conformational or non-linear antigenic sites. We should be able to confirm this when we complete our epitope scanning studies using solid-phase peptide synthesis on pin-supports, as developed by Geysen (38). Once the peptides are synthesized, we propose to examine the four MAbs as well as polyvalent Abs for reactivity. It seems likely that this approach will lead to a better understanding of crot toxin-antibody relationships.

The phospholipase active site region from a variety of different snake venom phospholipases A₂, including *C. atrox* and the basic subunit of crot toxin, shows absolute conservation, including four residues that form a 'catalytic network'. Yet we see no cross-reactivity between any of our monoclonal antibodies and phospholipase A₂ from *C. atrox*, indicating that the 'catalytic network' residues are not in the epitope recognized by our monoclonal antibodies.

It was surprising to find that both MAbs 1 and 2 showed similar levels of potent inhibition of crot toxin's phospholipase activity (Figs. 4 and 7A); but only MAb 1 exhibited neutralizing activity of toxicity. This inhibition was specific, since under our conditions phospholipase A₂ from *C. atrox* was unaffected (Fig. 6). Strong et al. (39) showed a maximum of 50% inhibition of phospholipase activity of β -bungarotoxin with non-neutralizing monoclonal antibodies raised against this toxin, results in keeping with what we found for our monoclonal antibodies 5 and 11.

It is clear that the presence of the acidic subunit enhances the inhibition of phospholipase activity associated with the basic subunit. That this inhibition is enhanced by addition of acidic subunit, suggests that there is association of the acidic subunit and phospholipase-containing basic subunit during phospholipid hydrolysis. What this association is and how it enhances the

extent of phospholipase inhibition by MAb 1 is presently unclear.

To define more completely the immunological relationships among phospholipase A₂ neurotoxins and enzymes, ten purified phospholipase A₂ neurotoxins and one cytotoxin from snake venoms were isolated and used to raise antisera in rabbits. Using ELISAs, we examined the cross-reactivities of the antisera and with the immunizing toxins and three additional toxins or enzymes. The proteins appeared to fall into two antigenic classes. One class was composed of the elapid toxins and pancreatic phospholipase A₂, while the other was made up of the viperid and crotalid proteins. The first class appears to have two subgroups, β -bungarotoxin and all the others. Likewise, one could designate two subgroups for the second class as the crotalid toxins or enzymes and the viper toxins. There appeared to be a limited or weak recognition of epitopes on elapid toxins by antisera to the crotalid and viperid toxins, while the converse was not observed. The exception to this rule was β -bungarotoxin, which was recognized by every antiserum we tested. We found that in general, there was a reciprocity seen with the cross-reactions, in that when antiserum to one toxin produced a moderate to high titer against another, the converse was also true. A practical issue raised by these results is whether the cross-reactivities identified by ELISA may reflect a potential for cross-neutralization. Work is currently in progress in Middlebrook's laboratory exploring this possibility.

Gel filtration and SDS-PAGE indicate that the crude venom of *C. d. collilineatus* contains a crotoxin-like material. The intact toxin and the subunits have LD₅₀ values which are comparable to those of crotoxin. The report by Faure and Bon (29), who found only minimal amounts of crotoxin-like material in venom from *C. d. collilineatus*, is difficult to reconcile. They may either have been looking at venom from a subspecies that simply did not contain the toxin or perhaps their venom supplier provided them with a miss-labeled sample.

It is difficult to explain the venom similarities between *C. atrox* and *C. s. scutulatus* (Type B) venom, and the profound, discrete differences between the two types of *C. s. scutulatus* venom. One possible explanation is that Type B venom snake populations have arisen in central Arizona through a series of recent hybridization events between *C. atrox* and *C. s. scutulatus*. While we are unaware of any published reports of wild-caught hybrids between these two species the absence of post-mating isolating mechanisms between the two species is clear, given the evidence presented in our paper (11).

The manuscript describing the amino acid sequence of the acidic subunit B-chain of crotoxin (16), completes our sequencing work on crotoxin. The sequence reported in the above paper is consistent with the final 24-residues reported earlier (2), except for position 20. Using automated Edman degradation we earlier found glycine at that position, whereas with mass spectrometry we found glutamic acid. Re-examination of the original Edman degradation results are consistent with the glycine identification. We can only attribute the difference to the presence of two different isoforms, one of which was sequenced by each of the methods. Bouchier et. al. (40) sequenced the cDNA encoding both subunits of crotoxin, including the B-

chain and found the sequence identical to that reported here, with only glutamic acid at position 20. These workers did identify glutamine at position 68, which presumably cyclizes to pyroglutamate in the mature protein. The B-chain reported here shows 91% identity with the comparable segment from Mojave toxin; 77% and 74% identity with the comparable segment of non-toxic phospholipase A₂ from the venom of *C. adamanteus* and *C. atrox*, respectively; and only 49% identity with that portion of the basic subunit chain of crototoxin.

It was tempting to interpret the differences in CD and fluorescence spectra on intact crototoxin and the sum of subunit spectra as evidence for a large conformational change upon complex formation between the acidic and basic subunits of all four proteins. Further studies showed however, that exposure of intact crototoxin to urea and subsequent removal by dialysis resulted in spectra (CD, FTIR, fluorescence) that were virtually identical to native intact crototoxin. This suggested that the changes seen in the spectra generated from reassociated, subunits, resulted from changes during subunit isolation and not merely from urea-induced conformational changes. What is suggested is that the mutual presence of the subunits is required for their proper folding upon removal of the denaturant, and reassembly into native oligomeric toxin. More definitive studies will require a subunit isolation procedure that does not alter subunit structure.

Fluorescence maxima of the tryptophans were not markedly shifted from subunits to intact toxins, even though there was a quenching of intrinsic fluorescence in intact material. This indicated that tryptophans remained in relatively polar environments in the dimeric toxins as well as in isolated subunits. While intact crototoxin, reconstituted from isolated subunits, exhibited obvious spectral differences from native crototoxin, they differed little in toxicity, suggesting that only limited regions of the protein are involved in these activities.

Because of currently accepted ancestral relationships among crotalids and reports of neurotoxicity in people bitten by *L. m. muta*, we were interested in examining its venom for crototoxin homologs. Using a combination of gel-filtration, SDS-PAGE, LD₅₀ assays and immunological assays, we found no evidence for a crototoxin homolog in the venom we examined. We did observe a high molecular weight protein that when injected i. v. into mice it induced effects characteristic of gyroxin, from *C. d. terrificus* venom. However, the Lachesis protein appears to be nearly twice as large as gyroxin (=60,000 vs 33,500) and is more toxic (LD₅₀ = 0.07 μ g/g), but promotes the same bizarre, rapid barrel-rolling motions. The amino acid sequence of its amino terminus shows considerable similarity to the amino terminus of gyroxin and other serine proteases, some of which are responsible for processing peptide hormones and clotting protein precursors. It would be interesting to know the distribution of this protein in different snake venoms and how it induces the barrel-rolling motion.

The water soluble EDC appears to covalently cross-link the two subunits of crototoxin. Recovered product has the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and a greatly reduced number of lysines. It has decreased immunological

cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crotoxin. This work is consistent with the results of Hendon and Tu (22), suggesting that crotoxin dissociation is required for expression of neurotoxicity. It does not however rule out the possibility that modification of essential residues result in the loss of toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crotoxin.

A sensitive, reliable method for the continuous measurement of acetylcholine and choline in in vitro reactions is sorely needed. We still feel that luminometry is the best procedure for obtain the sensitivity needed, but it is currently fraught with experimental difficulties that have yet to be resolved.

Results obtained in collaborative experiments with Lance Simpson's laboratory, which examined the interaction between crotoxin and the mammalian neuromuscular junction, suggested that crotoxin - but not the isolated subunit - binds avidly to the mammalian neuromuscular junction. The binding step is followed by a poisoning step, and there may be intermediate events as well. However, the data do not currently support the concept of a receptor-mediated endocytosis step that is interposed between binding and paralysis.

Venom fractionations were carried out using previously published procedures, for the most part. Our preparation of notexin from Notechis scutatus scutatus was the one major exception. We have spent considerable time in developing a fractionation procedure employing a combination of Sephadryl S-200HR followed by cation-exchange chromatography and another gel filtration step. Our final preparations of notexin and notechis II-5 appear homogeneous on SDS-PAGE and have i.v. LD₅₀-values of 0.005 and 0.04 µg/g, respectively. We have isolated what appears to be a new, toxic phospholipase in the process, which migrates just ahead of notexin on SDS-PAGE, cross-reacts with a MAb raised to pseudexin - as does notexin and notechis II-5 - and has an LD₅₀ = 0.008µg/g. Characterization of this product is continuing.

CONCLUSIONS

1. The four monoclonal antibodies and rabbit polyclonals raised against the basic subunit of crotoxin have been further characterized for their cross-reactivity against each other, in the first step of attempting to determine their binding epitopes. One of the monoclonal antibodies is a potent neutralizer of crotoxin's lethality and phospholipase activity. Another, is a non-neutralizer, but is able to inhibit the phospholipase activity by up to 90%. Work is continuing in an attempt to identify the neutralizing MAb epitope and other antigenic regions in the basic subunit of crotoxin recognized by the polyclonal antibodies.
2. A crotoxin homolog is present in the venom of C. v. concolor, C. s. scutulatus (Type A venom), C. vegrardis, and C. d. collilineatus, but none was found in venom from either C. v. lutosus, Lachesis muta muta, or C. atrox/C. atrox-C. s. scutulatus (Type B venom) hybrids.

With Lachesis we did demonstrate the presence of a toxic, 60 kd gyroxin-analog, which produced barrel-rolling motions in mice.

3. Determination of the primary sequences of the acidic and basic subunits of crototoxin have been completed.

4. Early structural studies on crototoxin and related crotalid dimeric, presynaptic neurotoxins indicated that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Additional studies showed however, that when intact crototoxin was exposed to urea and subsequently re-isolated, their spectra were indistinguishable from unexposed, intact crototoxin. Findings which suggest that the spectral changes observed in isolated subunits were artifacts, resulting from structural changes that occurred during subunit isolation by urea ion-exchange that was not easily reversed under our *in vitro* conditions.

5. Luminescence methodology still appears to be the most desirable method for choline and acetylcholine detection if the experimental problems can be resolved.

6. Collaborative efforts with Lance Simpson have provided us with another technique with which to examine the interaction of presynaptic neurotoxins with mammalian neuromuscular junctions. This methodology indicates that intact crototoxin, but not the individual subunits, bind with great affinity to the mammalian neuromuscular junction.

7. After unsuccessfully identifying any crototoxin basic subunit clones from our cDNA library, and acidic subunit clones with extensive sequence differences, we concluded that the preparation of new libraries were in order. Both cDNA and genomic libraries from C. s. scutulatus are in preparation.

8. Crototoxin cross-linked by a different cross-linker than used by He on and Tu, also proved to be non-toxic. These results do not rule out the possibility that modification of essential residues result in the loss of the observed toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crototoxin.

9. In our purification of toxins, we developed an updated purification procedure for notexin and notechis II-5 from N. s. scutatus. In addition, we appear to have isolated a new, toxic phospholipase with an LD₅₀ = 0.008μg/g in mice.

RECOMMENDATIONS

1. Continue the characterization of the monoclonal antibodies and attempt to identify the epitope recognized by the neutralizing monoclonal antibody. Also attempt to identify antigenic regions recognized by the polyclonal antibodies.
2. Continue our collaboration with Dr. Keith Ward on the determination of the crystal structure of Mojave toxin that is in progress. Expand this collaboration when additional purified toxins and subunits become available.

3. Continue attempts to establish an in vitro system using synaptosomes and bioluminescence for examining the biological effects of presynaptic neurotoxins on acetylcholine release. Maintain the collaboration with Dr. Lance Simpson, whose phrenic nerve-hemidiaphragm system may help elucidate the mechanism of action of the phospholipase A₂ neurotoxins.
4. Continue to characterize the cDNA and genomic clones of C.s. scutulatus, and attempt to get them expressed for future site-specific mutagenesis studies.
5. Further characterize the new toxic phospholipase A₂ found in the venom of N. scutatus during the purification of crotexin and notechis II-5.

LITERATURE CITED

1. Slotta, K. H. and Fraenkel-Conrat, H. (1938) Schlangengifte III. Mitt. Reinigung und Kristallisation des Klapperschlangen-gifles. Ber. dt chem. Ges. 71, 1076-1081.
2. Aird, S. D., Kaiser, I. I., Lewis, R. V., and Krugel, W. G. (1985) Rattlesnake presynaptic neurotoxins: primary structure and evolutionary origin of the acidic subunit. *Biochemistry* 24, 7054-7058.
3. Hendon, R. A. and Bieber, A. L. (1982) Presynaptic toxins from rattlesnake venoms. In: *Rattlesnake Venoms: Their Actions and Treatment*, p. 211 (Tu, A. T., Ed.) New York: Marcel Dekker.
4. Fraenkel-Conrat, H. (1983) Snake venom toxins related to phospholipase A₂. *J. Toxicol.-Toxin Reviews* 1, 205-221.
5. Aird, S. D. and Kaiser, I. I. (1985) Comparative studies on three rattlesnake toxins. *Toxicon* 23, 361-374.
6. Kaiser, I. I., Middlebrook, J. L., Crumrine, M. G., and Stevenson, W. W. (1986) Cross-reactivity and neutralization by rabbit antisera raised against crot toxin, its subunits, and two related toxins. *Toxicon* 24, 669-678.
7. Kaiser, I. I. and Middlebrook, J. L. (1988) Preparation of a crot toxin neutralizing monoclonal antibody. *Toxicon* 26, 855-866.
8. Kaiser, I. I. and Middlebrook, J. L. (1988) Comparative properties of a neutralizing and three non-neutralizing monoclonal antibodies to crot toxin. In *Neurotoxins in Neurochemistry* (Dolly, J. O., Ed.), pp. 43-51, Ellis Horwood, Chichester.
9. Kaiser, I. I. and Aird, S. D. (1987) A crot toxin homolog from the venom of the Uracoan rattlesnake (Crotalus vegrandis). *Toxicon* 25, 1113-1120.
10. Aird, S. D., Seebart, C. S., and Kaiser, I. I. (1988) Preliminary fractionation and characterization of the venom of the great basin rattlesnake (Crotalus viridis lutosus). *Herpetologica* 44, 71-84.

11. Aird, S. D., Thirkhill, L. J., Seebart, C. S., and Kaiser, I. I. (1989) Venoms and morphology of Western diamondback/Mojave rattlesnake hybrids. *J. Herpetology* 23, 131-141.
12. Fraenkel-Conrat, H., Jeng, T. W., and Hsiang, M. (1980) Biological activities of crototoxin and amino acid sequences of crototoxin B. In: *Natural toxins*, p. 561 (Eaker, D. and Wadstrom, T., Eds.) New York: Pergamon.
13. Aird, S. D., Kaiser, I. I., Lewis, R. V., and Kruggel, W. G. (1986) A complete amino acid sequence for the basic subunit of crototoxin. *Arch. Biochem. Biophys.* 249, 296-300.
14. Aird, S. D., Kaiser, I. I., Lewis, R. V., and Kruggel, W. G. (1987) A complete amino acid sequence for the basic subunit of crototoxin. *Toxicon* 25, 113.
15. Aird, S. D., Yates, R. J. III, Hunt, D. F., and Kaiser, I. I. (1989) Amino acid sequence of crototoxin's acidic subunit B chain. *Toxicon* 27, 29.
16. Aird, S. D., Yates, J. R. III, Martino, P. A., Shabanowitz, J., Hunt, D. F., and Kaiser, I. I. (1989) The amino acid sequence of the acidic subunit B chain of crototoxin (submitted).
17. Hanley, M. R. (1979) Conformation of the neurotoxin crototoxin complex and its subunits. *Biochemistry* 18, 1681-1688.
18. Tu, A. T., Fox, J. W., Pool, W. R., and Bieber, A. L. (1982) Comparison of conformations of Mojave toxin subunits and reconstituted toxin. *Comp. Biochem. Physiol.* 73B, 459-464.
19. Dufton, M. J., Eaker, D., and Hider, R. C. (1983) Conformational properties of phospholipases A₂. *Eur. J. Biochem.* 137, 537-544.
20. Aird, S. D., Steadman, B. L., Middaugh, C. R., and Kaiser, I. I. (1989) Comparative spectroscopic studies of four crototoxin homologs and their subunits. *Biochim. Biophys. Acta* 997, 211-218.
21. Norden, T. M., Ward, K. B., Kaiser, I. I., and Aird, S. D. (1987) Crystals of Mojave toxin. *Toxicon* 25, 149-150.
22. Hendon, R. A. and Tu, A. T. (1979) The role of crototoxin subunits in tropical rattlesnake neurotoxic action. *Biochim. Biophys. Acta* 578, 243-252.
23. Rosenberg, P., Condrea, E., Rapuano, B. E., Soons, K. R., and Yang, C. C. (1983) Dissociation of pharmacological and enzymatic activities of snake venom phospholipase A₂ by modification of carboxylate groups. *Biochem. Pharmacol.* 32, 3525-3530.
24. Jeng, T. W. and Fraenkel-Conrat, H. (1978) Chemical modification of histidine and lysine residues of crototoxin. *FEBS Lett.* 87, 291-296.
25. Parsons, T. F. and Pierce, J. G. (1979) Biologically active covalently cross-linked glycoprotein hormones and the effects of modification of the COOH-terminal region of their a subunits. *J. Biol. Chem.* 254, 6010-6015.
26. Carraway, K. L. and Koshland, D. E. (1968) Reaction of tyrosine residues in proteins with carbodiimide reagents. *Biochim. Biophys. Acta* 160, 272-274.

27. Goncalves, J. M. (1956) Purification and properties of crotamine. In:Venoms, p. 261 (Buckley, E. E. and Porges, N., Eds.). American Assn. Adv. Sci., Washington, D.C.
28. Ownby, C. L., Cameron, D. and Tu, A. T. (1976) Isolation of myotoxic components from rattlesnake (*Crotalus viridis viridis*) venom. Electron microscopic analysis of muscle damage. Am. J. Path. 85, 149-158.
29. Faure, G. and Bon, C. (1987) Several isoforms of crototoxin are present in individual venoms from the South American rattlesnake *C. d. terrificus*. Toxicon 25, 229-234.
30. Trivedi, S., Kaiser, I. I., Tanaka, M., and Simpson, L. L. (1989) Pharmacologic Experiments on the interaction between crototoxin and the mammalian neuromuscular junction. J. Pharmacol. Exper. Ther. (in press).
31. Bio-Rad Laboratories, Instruction Manual (86-0513 1286) included with Immuno-Blot Assay Kit, Goat anti-mouse alkaline phosphatase conjugate.
32. Middlebrook, J. L. and Kaiser, I. I. (1989) Immunological relationships of phospholipase A2 neurotoxins from snake venoms. Toxicon 27, 965-977.
33. Aird, S. D., Steadman, B. L., Middaugh, C. R., and Kaiser, I. I. (1988) Spectroscopic studies of crototoxin homologs and their subunits. Federation Proceedings 2, A1352.
34. Ownby, C. L., Aird, S. D., and Kaiser, I. I. (1988) Physiological and immunological properties of small myotoxins from the venom of the midget faded rattlesnake (*Crotalus viridis concolor*). Toxicon 26, 319-323.
35. Da Silva, N. J., Aird, S. D., Seebart, C., Kaiser, I. I. (1989) A gyroxin analog from the venom of the Bushmaster (*Lachesis muta muta*). Toxicon, 27, 763-771.
36. Lennon, B. W., Plummer, D., and Kaiser, I. I. (1989) Effects of subunit cross-linking on the properties of crototoxin (in press).
37. Israel, M. and Lesbats, B. (1987) The use of bioluminescence techniques in neurobiology, with emphasis on the cholinergic system. In Neurochemistry, A. J. Turner and H. S. Bachelard, Eds., IRL Press, Washington, DC, pp. 113-136.
38. Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbick, G., and Schoofs, P. G. (1987) Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods 102, 259-274.
39. Strong, P. N., Wood, J. N., and Ivanyi, J. (1984) Characterization of monoclonal antibodies against β -bungarotoxin and their use as structural probes for related phospholipase A₂ enzymes and presynaptic phospholipase neurotoxins. Eur. J. Biochem. 142, 145-151.
40. Bouchier, C., Ducancel, F., Guignery-Frelat, G., Bon, C., Boulian, J. C., and Menez, A. (1988) Cloning and sequencing of cDNAs encoding the two subunits of crototoxin. Nucleic Acids Research 16, 9050.

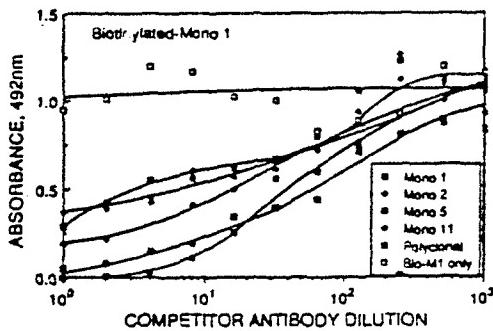


Fig. 1. Competition of MAbs and polyclonal Abs for the binding of biotinylated-MAb 1 to the basic subunit of crot toxin. Microtiter wells coated with the basic subunit of crot toxin were incubated with decreasing concentrations of competitor Abs in the presence of biotinylated-MAb 1. Bound biotinylated-MAb was detected by an avidin-biotin-peroxidase complex as described (Kaiser and Middlebrook, 7). Concentration of biotinylated-MAb was 50 ng/well and initial unlabeled Ab concentration was 3 µg/well.

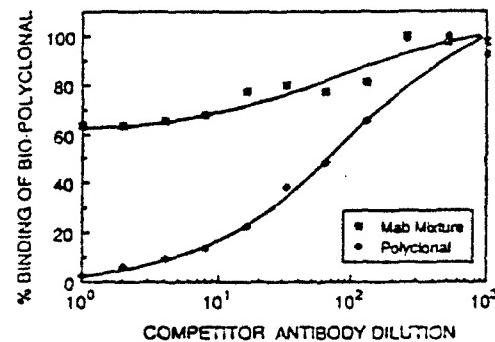


Fig. 2. Competition of a mixture of four MAbs (raised against the basic subunit of crot toxin) for the binding of biotinylated-polyclonal Abs (raised in a rabbit against the basic subunit of crot toxin). Well coating, dilutions, and detection were as described in legend for Fig. 1. Concentration of biotinylated-polyclonal antibodies was 25 ng/well and the initial concentrations of polyclonal antibodies and the MAb mixture were 3 and 12 µg/well, respectively.

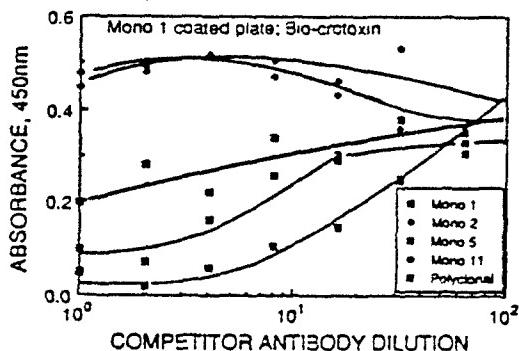


Fig. 3. Competition of MAbs and polyclonal Abs for the binding of biotinylated-crotoxin to wells coated with MAb 1. Biotinylated-crotoxin is mixed with different dilutions of the test Ab and incubated overnight. The mixtures are then added to wells coated with MAb 1. Lack of competition between the test Ab and MAb 1 for the same epitope should not affect biotinylated-crotoxin binding. Bound biotinylated-crotoxin was detected as in Fig. 1. Biotinylated crotoxin was 50 ng/well and initial concentrations of mono- and polyclonal antibodies were 1 and 3 µg/well, respectively.

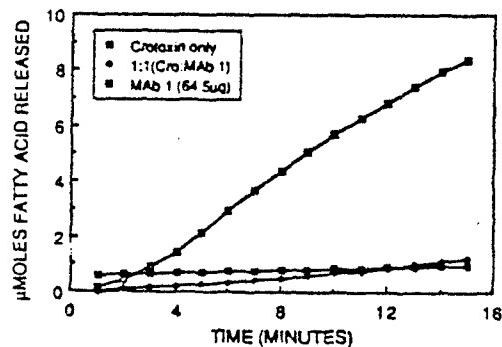


Fig. 4. Phospholipase activity of crotoxin on phosphatidylcholine at a 2:1 molar ratio of Triton X-100-phospholipid in the presence and absence of MAb 1. Fatty acids released were titrated and maintained at pH 8 with dilute NaOH under nitrogen at 37°, using a radiometer titration apparatus (Aird and Kaiser, 5). One ml of substrate was used per reaction. Phospholipase activity was assayed after the addition of 10 µg of crotoxin and any MAbs at the indicated concentration. When Abs were used, they were preincubated with the phospholipase for 15 min at 37° before being assayed unless indicated.

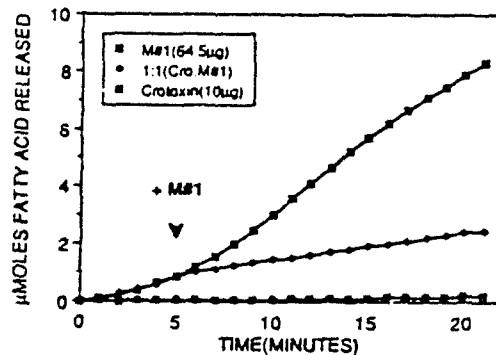


Fig. 5. Phospholipase activity of crot toxin in the absence and presence of MAbs 1 added 5 min into the reaction. Other conditions as described as in Fig. 4.

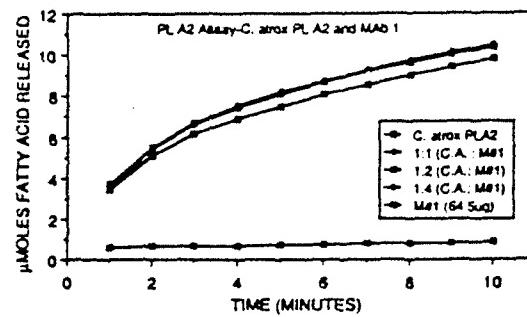


Fig. 6. Phospholipase activity of phospholipase A₂ from *C. atrox* on phosphatidyl choline in the absence and presence of different concentrations of MAbs 1. Other conditions as described in Fig. 4.

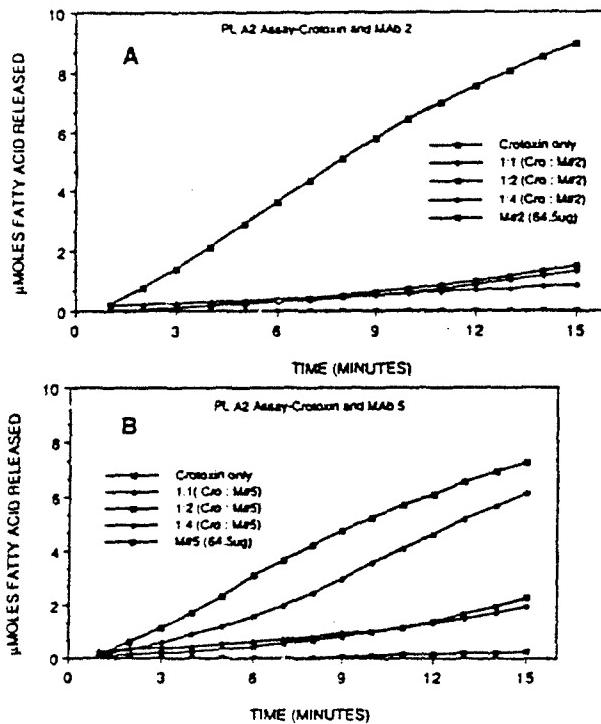


Fig. 7. Phospholipase activity of crot toxin in the absence and presence of different concentration of (A) MAbs 2 and (B) MAbs 5. Other conditions as described in Fig. 4.

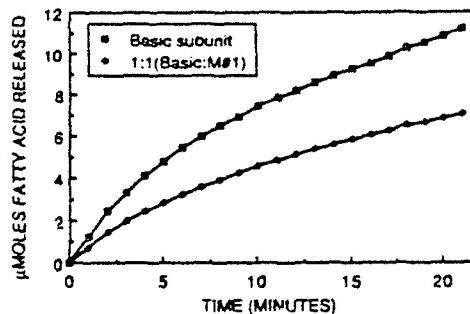


Fig. 8. Phospholipase activity of the basic subunit of crot toxin (6.2μg) on phosphatidyl choline in the absence and presence of an equal molar amount of MAbs 1. Other conditions as described in Fig. 4.

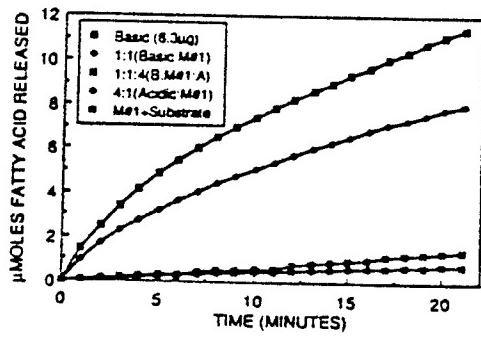


Fig. 9. Phospholipase activity of the basic subunit of crot toxin (6.3μg) in the absence and presence of equal molar amounts of MAb 1; and in the presence of both MAb 1 and acidic subunit of crot toxin. Other conditions as described in Fig. 4.

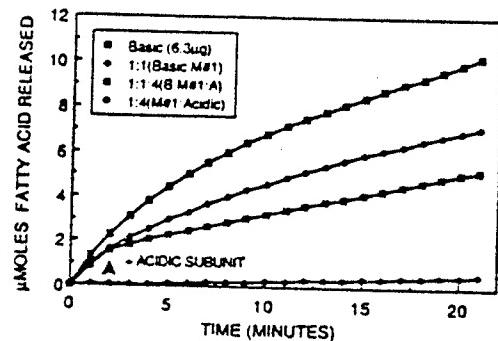


Fig. 10. Phospholipase activity of the basic subunit of crot toxin (6.3μg) in the absence and presence of equal molar amounts of MAb 1. In one reaction a four-fold excess of the acidic subunit was added to a basic subunit-MAb 1 mixture. Other conditions as described in Fig. 4.

TABLE A. BIOTINYLATED-MONOCLONAL ANTIBODY

BIO-Ab	1	2	5	11	P
1	++++	++	++	+++	+++++
2	+	++++	+	+++	+++++
5	+++	+	++++	+	+++++
11	+	++++	+	++++	+++++

COMPETITOR ANTIBODY

TABLE B. BIOTINYLATED-CROTOXIN

Ab COATING PLATE	1	2	5	11	P
1	++++	0	++	0	+++
2	0	++	+	++++	+++
5					
11	0	++++	++	+++++	+++

COMPETITOR ANTIBODY

Table 1. Summary and semiquantitation of the data showing the competition of (A) different antibodies with biotinylated-MAb for immobilized basic subunit of crot toxin; and (B) biotinylated-crot toxin for immobilized MAbs.

DISTRIBUTION LIST

- 5 copies Commander
US Army Medical Research Institute of Infectious Disease
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011
- 1 copy Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick, Frederick, MD 21701-5012
- 2 copies Defense Technical Information Center (DTIC)
ATTN: DTIC-FDAC
Cameron Station
Alexandria, VA 22304-6145
- 1 copy Dean
School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799
- 1 copy Commandant
Academy of Health Sciences, US Army
ATTN: AHA-CDM
Fort Sam Houston, TX 78234-6100